Chapter 1

The Role of Androgens in Ovarian Follicular Development: From Fertility to Ovarian Cancer

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Abstract

Androgens, steroid hormones produced by follicular cells, play a crucial role in the regulation of ovarian function. They affect folliculogenesis directly through androgen receptors (ARs) or indirectly through aromatization to estrogens. Androgens are thought to be primarily involved in preantral follicle growth and prevention of follicular atresia. It also seems possible that they are involved in the activation of primordial follicles. According to the World Health Organization, endocrine-disrupting chemicals (EDCs) are substances that alter hormonal signaling. EDCs comprise a wide variety of synthetic or natural chemicals arising from anthropogenic, industrial, agricultural, and domestic sources. EDCs interfere with natural regulation of the endocrine system by either mimicking or blocking the function of endogenous hormones as well as acting directly on gene expression or through epigenetic modifications. Disruptions in ovarian processes caused by EDCs may originate adverse outcomes such as anovulation, infertility, or premature ovarian failure. In this chapter, we aim to point out a possible involvement of androgen excess or deficiency in the regulation of ovarian function. We will summarize the effects of EDCs expressing antiandrogenic or androgenic activity on female physiology. Continuous exposition to even small concentration of such compounds can initiate oncogenesis within the ovary.

Keywords: androgens, androgen receptors, ovarian follicle, folliculogenesis, endocrine-disrupting chemicals

1. Introduction

The mammalian ovarian follicle guarantees two essential functions in the ovary. It synthesizes many substances, including steroids, and by this way creates a microenvironment for the proper development and maturation of a viable oocyte. Even though gonadotrophins are

regarded as the main hormones regulating follicular development, sex steroids are also known to play an important role in this process. Currently, the least established follicular function is that related to androgens. Androgens were originally regarded as hormones influencing primarily the male physiology. This perception has changed as numerous investigations have demonstrated the effects of androgens such as testosterone (T) and dihydrotestosterone (DHT) on female physiology [1]. It turned out that androgens are one of the most important agents influencing folliculogenesis [2–6]. Androgens are known to exert pro-apoptotic effects [7, 8] but are also indispensable in normal folliculogenesis for both androgen receptor-mediated responses and as substrates for estrogen synthesis [9]. Androgenic actions play a role mainly in early follicular growth, whereas estrogenic roles are more important at later follicle development stages [1, 9]. The high number of androgen receptors (ARs) that characterize granulosa cells (GCs) in preantral follicles declines during antral differentiation at the same time as expression of mRNA for P450 aromatase (P450arom) and estrogen synthesis increase [10–13].

Recently, a growing concern aroused about the potential for environmental endocrine-disrupting chemicals (EDCs) to alter sexual differentiation. EDCs are one of the factors that can induce unfavorable changes taking place in the ovary [14, 15]. They originate as a result of human industrial activities, enter the natural environment, and then disturb hormonal regulation (e.g., through blocking steroid hormone receptors) [16]. Such a mechanism of action negatively influences many processes taking place in the reproductive tract of a female [17, 18]. In extreme cases, this may lead to the elimination of many populations from their natural habitats, by premature cessation of ovarian function, among other putative mechanisms. The image of muscular bodies as the model for an ideal, which is frequently carried in mass communication media, has led to an increase in the number of enthusiasts for androgenic anabolic steroid (AAS) use. AAS is a group of synthetic compounds that originate from testosterone and its esterified or alkalinized derivatives belonging to EDCs. The association between AAS use and cancer that has been described in the literature and may be related to the genotoxic potential has already been shown in several studies [19, 20]. *In vitro* toxicological models are widely used to assess the effects of endogenous androgens and EDCs on ovarian function, to understand their role in the initiation/progression of ovarian cancers.

In this chapter, we intend to point out a possible impact of androgen excess or deficiency on the regulation of ovarian function as well as following EDC action with antiandrogenic (e.g., vinclozolin, linuron) or androgenic (e.g., anabolic steroids: testosterone propionate, boldione) activity due to the fact that continuous exposition to even small concentration of such compounds can initiate oncogenesis within the ovary. Following our previous results obtained using an in vitro animal model generated for studying androgen deficiency, we have found that the exposure of porcine follicles to an environmental antiandrogen—vinclozolin—caused deleterious effects at antrum formation stage that may negatively influence the reproductive function in mammals.

2. Androgen receptor structure and mechanism of action

Like all steroid hormones, androgens affect target cells by binding to and activating specialized receptors. The types of receptors that are involved in the signal transduction decide on

its mechanism of action. A genomic response is usually induced by receptors localized in the cytoplasm/nucleus. Additionally, androgens can also exert their effects by interacting with receptors located on the cell membrane to perform rapid, non-genomic actions. It is well known that the cross talk between non-genomic and genomic signaling pathways is crucial for proper ovarian function [21].

The ARs, encoded by a gene composed of eight exons located on the X chromosome, are proteins with approximately 919 amino acids. The exact length of ARs is variable due to the existence of two diverse polyglutamine and polyglycine stretches in the N-terminal region of the protein [22]. This AR region modulates its transactivation [23, 24] and, hence, its functionality. The ARs, which belong to the nuclear receptor superfamily, are characterized by a modular structure consisting of four functional domains: C-terminal domain responsible for ligand binding (LBD), a highly conserved DNA-binding domain (DBD) with centrally located zinc fingers, a hinge region, and N-terminal domain (NTD) (**Figure 1**) [25, 26]. The C-terminal domain of ARs is encoded by exons 4–8. Within itself, besides LBD, C-terminal domain also contains transcriptional activation function 2 (AF2) co-regulator binding interface [27, 28]. In the most conserved region of ARs—DNA-binding domain—two zinc fingers encoded by exon 2 and exon 3, respectively, are located. The first zinc finger determines the specificity of DNA recognition, which makes contact with major groove residues in an androgen-response element (ARE) half-site. The second zinc finger is a dimerization interface that mediates binding with a neighboring AR molecule engaged with an adjacent ARE half-site [29]. The short flexible hinge region, encoded by exon 4, regulates DNA binding, nuclear translocation, and transactivation of the ARs [30]. The N-terminal domain, encoded by AR exon 1, is relatively long and poorly conserved. It displays the most sequence variability by, as mentioned above, virtue of polymorphic (CAG)n and (GGN)n repeat units encoding polyglutamine and polyglycine tracts, respectively [31–33]. This domain contains also the AF1, which harbors two transactivation regions, transcriptional activation unit-1 (TAU-1), and transcriptional activation unit-5 (TAU-5). The N-terminal domain is essential for AR activation [34] and, because it contains many sites for Ser/Thr phosphorylation, may be involved in mediating cross talk with other signaling pathways leading to the modulation of AF1 activity and interaction with co-regulators [35].

In the absence of androgens, unliganded ARs remain in the cytoplasm. To maintain the unbounded AR protein in a stable and inactive configuration, the molecular chaperone complex, including Hsp90 and high-molecular-weight immunophilins, is needed. Androgens like other steroids can freely diffuse across the plasma membrane and bind to the LBD region that induces conformational changes, including the Hsp90 dissociation from ARs. Followed by these transformation, ARs undergo dimerization, phosphorylation, and translocation to the nucleus, which is mediated by the nuclear localization signal (NLS) in the hinge region. The dimer binds to the androgen response elements (AREs) located in the promoter of the target gene and leads to the recruitment of co-regulators, either coactivators or corepressors such as steroid receptor coactivator 1 (SRC1) and transcriptional intermediary factor 2 (TIF2), leading to transcription of genes that are involved in many cellular activities, from proliferation to programmed cell death [36]. In some cases, for example, in the low androgen concentration, the ligand-independent signaling pathway may occur. This process involves MAPK/ERK pathway and depends on growth factor

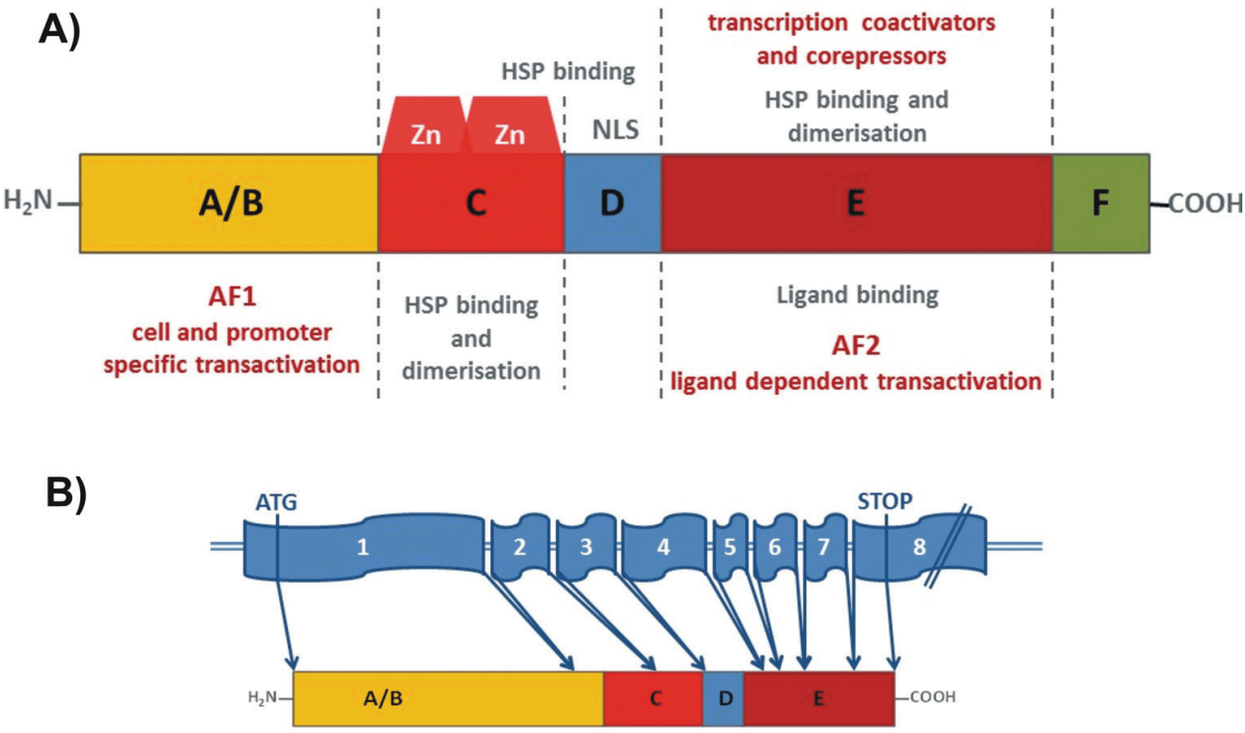

Figure 1. Schematic representation of the structural and functional domains of AR protein (**A**) and the coding of exons 1–8 in relation to each functional domain of human AR gene (**B**). AF, transcriptional activation function; NLS, nuclear localization signal; HSP, heat shock protein.

receptors. As a result, transcriptional activity enhancement, through direct phosphorylation of steroid receptors, is observed [37]. The androgen signaling pathways depicted above are collectively known as "genomic pathway" (**Figure 2**) [38].

Apart from the direct or indirect genomic effects, androgens may also operate in cells by the "non-genomic pathway," stimulating rapid effects in signal transduction through the production of second messengers, ion channel transport, and protein kinase cascades. This kind of activity involves receptors localized in the plasma membrane or in "lipid rafts" [39]. Rapid non-genomic action of androgens might be mediated by binding to transmembrane receptors unrelated to nuclear hormone receptors (usually G-protein-coupled receptor (GPCR)) that was well documented in different tissues [40, 41]. Among GPCRs, there are GPRC6A and ZIP9 that have been pharmacologically well characterized [42, 43]. Additionally, androgens can induce activation of the Src/Ras/Raf/MAPK/ERK1/ERK2 pathway in the cytoplasm, independently of receptor-DNA interactions (**Figure 2**) [44, 45]. It was shown that in luteinized human GCs androgens caused rapid, non-genomic-dependent rise in cytosolic calcium, involving voltage-dependent calcium channels in the plasma membrane and phospholipase C [46, 47].

Androgen action might be disturbed by alternative splicing [48]. This is a common event described in the structural molecular biology of AR genes. Alternative splicing is a process by which multiple different mRNAs and downstream proteins can be generated from one gene through the inclusion or exclusion of specific exons [49]. This process might occur in

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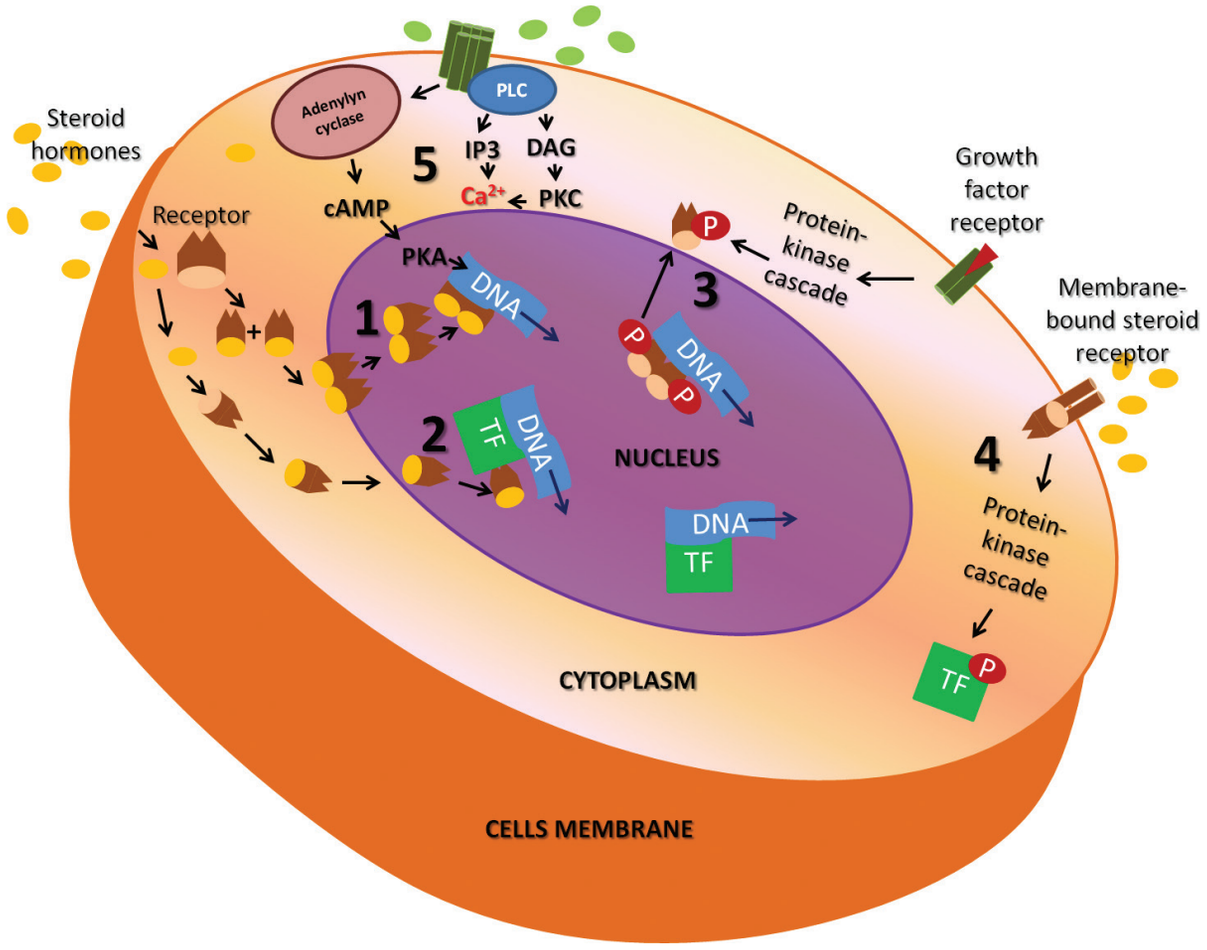

Figure 2. Molecular mechanism of the AR action. After entering into the cell, ARs bind to their specific receptors located in the cytoplasm; the ligand-receptor complexes are then translocated to the nucleus. After that, they bind to DNA as dimmers modulating gene expression (**1**). Alternatively, the ligand-receptor complexes in the nucleus interact with transcription factors, which in turn bind to their responsive elements on the DNA to regulate gene expression (**2**). Hormone-independent mechanism involves AR phosphorylation and activation, which is triggered by protein kinase cascade in response to growth factors binding to their receptors located on the cell membrane. Phosphorylated ARs enter the nucleus and bind to DNA, regulating gene expression (**3**). Androgens may also be directly bounded by cell membrane receptors, triggering the activation of protein kinase cascades. Thereafter, phosphorylated transcription factors bind to their own response elements in the genome, thereby controlling gene expression (**4**). Androgen action might be either mediated by intracellular secondary messengers produced in response to the activation of G-protein-coupled receptors (**5**). TF, transcription factor; cAMP, cyclic AMP; PKA, protein kinase A; PLC, phospholipase C; IP_3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C.

95% of all multi-exonic genes and provides a significant advantage in evolution by increasing proteomic diversity [50]. Although deregulation of this process may lead to inappropriate spliced mRNA, impaired proteins and eventually to diseases such as cancers [51, 52] or endocrine system dysfunction [53]. More recently, two AR splice variants expressed in GCs from patients with polycystic ovary syndrome (PCOS), which is one of the most common causes of female infertility, have been identified [54]. The altered AR splicing patterns are strongly associated with hyperandrogenism and abnormal folliculogenesis in PCOS [55]. It seems possible that AR alternative splicing may be an important pathogenic mechanism in human infertility.

3. Androgens and follicular development

In the ovary of a mature mammalian female, the process of folliculogenesis proceeds all the time, which manifests in cell proliferation and differentiation. Such a process, involving growth and development of ovarian follicles from the stage of primordial to the preovulatory ones, is a substantially complicated phenomenon requiring multidirectional regulation. From the initial pool of ovarian follicles starting to grow, the preovulatory stage is reached by only a few. More than 99% of the follicles undergo atresia at various stages of development. The transition from the preantral to an early antral stage is most susceptible to this process. All primordial follicles present during fetal life constitute a reserve that cannot increase later on, during the postnatal period. Therefore, the very first stages of folliculogenesis, such as formation of primordial follicles, their recruitment from the resting pool, and then transformation into primary ones, are critical for the reproductive cycle of a vertebrate female animal [56]. Improper coordination of the primordial follicle formation and activation of their growth may disturb folliculogenesis in mature individuals originating infertility.

3.1. Origin of primordial follicles

In the developing ovary, the primordial follicles consist of an oocyte surrounded by a single layer of squamous pregranulosa cells. Once assembled, some of the primordial follicles are immediately stimulated to growth, but most remain quiescent until selected follicles are gradually recruited into a growing follicle pool, throughout the reproductive life [57]. The recruitment of primordial follicles into a growth (primordial-to-primary follicle transition) involves a change in the shape of the granulosa cells from squamous to cuboidal and the initiation of oocyte growth. The primordial-to-primary follicle transition is an irreversible process. The early stages of folliculogenesis are believed to be gonadotropin independent. All events related to early follicular development are mostly regulated by paracrine growth factors originating from the growing oocyte itself and from the somatic cells that surround it [58, 59] and also by ovarian steroid hormones (i.e., progesterone, androgens, and estrogens) [6]. Interestingly, during initiation of primordial follicle growth, a fundamental role for androgens has been shown. In mouse, bovine and primate ovaries T and DHT [3, 60, 61] are responsible for the stimulation of this process, while in sheep DHEA plays the main role [62]. The initiation of primordial follicle growth might be mediated through paracrine stimulation, by upregulation of IGF-1 and/or its receptor [63]. On the other hand, it seems possible that androgens, acting through ARs, regulate the early stages of follicular development. Fowler et al. [61] reported that in human fetal ovaries pregranulosa cells express ARs, and the oocytes of the primordial follicles are able to synthesize androgens. Taken together, androgens might stimulate the primordial-to-primary follicle transition but still an open-ended question is that how they exactly influence primordial follicle recruitment and whether this is a primary or secondary response [64].

3.2. Antral follicle formation

Studies indicating AR expression in the different compartments of follicles throughout most stages of folliculogenesis allowed us to assume that androgens regulate follicular development [9].

Although AR expression pattern differs between follicular cell types, it has been observed that AR number declines together with follicle maturation to the preovulatory stage [65]. AR-mediated actions might be important in the antrum formation during follicular development. Mouse preantral follicles cultured in vitro in the presence of an AR antagonist, bicalutamide, showed significantly suppressed growth and antral cavity formation. At the same time, supplementation of culture medium with DHT restored the follicular growth and antral development in follicles cultured without FSH addition [66]. Similar situation was observed after different androgens (incl. T, DHT, or DHEA) in addition to in vitro culture system of mouse preantral follicles. They undergone rapid granulosa cell proliferation and amplified responsiveness to FSH [67]. Moreover, supplementation of culture media with estrogens, with or without fadrozole (an aromatase inhibitor), had no effect on follicular development, while the addition of an AR antagonist, flutamide, suppressed follicular growth. These studies allow to state that these androgen stimulatory effects on antrum formation and follicular growth are mediated directly through ARs and are not induced by T aromatization to estrogens [3]. Our recent research was conducted to determine whether experimentally induced androgen deficiency during in vitro culture of porcine ovarian cortical slices affects preantral follicular development. Cultured preantral follicles were supplemented with testosterone, nonsteroidal antiandrogen, 2-hydroxyflutamide, and a dicarboximide fungicide, separately or in combination with androgen. 2-Hydoxyflutamide is a pharmaceutical compound, which is regarded as a model antiandrogen in experimental studies. It promotes AR translocation to the nucleus and DNA binding but nevertheless fails to initiate transcription, inhibiting the AR signaling pathway [68]. We demonstrated the deleterious effects of androgen deficiency at antrum formation stage, what confirms androgen involvement in porcine early follicular development [69]. In summary, it was clearly shown that androgens enhance ovarian follicle growth, from preantral to antral stage. The main findings regarding the direct action of androgens on the in vivo and in vitro control of follicular development in mammals are based on the transcriptional actions of ARs in follicular cells.

3.3. Preovulatory follicular development

During antrum formation GCs separate into cumulus GCs and mural GCs, which line the follicle wall. These two subpopulations of GCs gain different morphological and functional properties during further follicle development [70]. The mural granulosa cells are characterized by high levels of steroidogenic enzyme activity, which converts androgens to estrogens, while the cumulus cells (CCs) are engaged in supporting oocyte growth and maturation. Just before ovulation, CCs acquire steroidogenic abilities and start to produce primarily progesterone [71]. The role of ARs in the female was elucidated by the studies of various global and tissue-specific AR knockout (ARKO) mouse models [72]. Granulosa cell-specific ARKO (GCARKO) mouse models have demonstrated that granulosa cells are an important site for androgen action and strongly suggested that the AR in these cells is an important regulator of androgen-mediated follicular growth and development. On the other hand, AR inactivation in the oocyte, as shown in the OoARKO female mouse model, appears to have no major overall effect on female fertility [73]. Using female mice lacking functional ARs (AR−/α), Hu et al. [74] demonstrated impaired expression of ovulatory genes, defective morphology of the preovulatory cumulus oophorus cells, and markedly reduced fertility. However, there are contradictory reports

concerning androgen effects on oocyte maturation and embryonic development. While some authors found androgens exerting inhibitory effects on these processes in different species [75, 76], others have shown that T increases the cleavage rate of fertilized rat oocytes and that dihydrotestosterone improves the fertilizability of mouse oocytes [77, 78]. Optimal androgen levels appear to be of real importance in the maintenance of proper preovulatory follicular development ensuring normal ovulatory function. Administration of T or DHT did not increase preovulatory follicle numbers in primate ovaries [12]. Yet, in pigs, treatment with T or DHT during the late follicular phase increased the number of preovulatory follicles and corpora lutea [79]. In mice, DHT at a low dose [80] improved the ovulatory response to superovulation. Likewise, in vivo treatment of rats with a steroidal AR blocker (cyproterone acetate) leads to a decrease in the number of new corpora lutea, indicating an inhibition of ovulation [81]. To sum up, these findings indicate that androgens indeed play a role at the preovulatory stage of follicle life cycle. Moreover, the coordination of oocyte maturation and ovulation is reactive to the androgenic environment. Therefore, a balance of androgen positive and negative actions is required for optimal ovarian functioning. Some contradictory findings on the role played by androgens in this period of follicle development stress the need for further research aimed at elucidating the background of these processes.

4. Antiandrogenic and androgenic EDC action within the ovary

In the light of a dramatic increase of evidences demonstrating the harmful effects of EDCs present in the environment, it is crucial for further research on the female reproductive potency to understand the mechanisms of their action within ovaries. Among EDCs there is a large group of chemicals exerting antiandrogenic effects and blocking endogenous androgen action. We can find there pharmaceuticals (e.g. 2-hydroxyflutamide, ketoconazole) as well as environmental contaminants: pesticides (e.g. vinclozolin, linuron) or synthetic androgens such as testosterone propionate or boldione, which are widely used anabolic steroids [82]. During our previous experiments concerning the involvement of androgen in ovarian follicular development and atresia, we generated an in vitro toxicological model for studying androgen deficiency. Using 2-hydroxyflutamide, which is a nonsteroidal antiandrogen acting at the AR level, we induced distortions of androgen action in the ovary that in consequence reduced porcine GC viability and proliferation [83].

Vinclozolin, a commonly used dicarboximide fungicide, is registered in the USA and Europe to prevent decay of fruits and vegetables. It was shown that vinclozolin possesses an antiandrogenic activity in mammals and fish [84–86]. Two major ring-opened metabolites of vinclozolin (butenoic acid M1 and enanilide M2) have been detected in rodent fluids and tissue extracts following in vivo exposure that might have negative consequences for human health [87–89]. Exposure to vinclozolin during gonadal sex determination period in mice promotes a transgenerational increase in pregnancy abnormalities and female adult onset malformation in the reproductive organs [90, 91]. Our previous studies showed that vinclozolin at an environmentally relevant concentration might contribute to the amplification and propagation of apoptotic cell death in the granulosa layer, leading to the rapid removal of atretic follicles

in porcine ovary [92, 93]. Besides, it seems possible that vinclozolin activates non-genomic signaling pathways directly modifying the AR action. Another widely used pesticide with antiandrogenic activity is linuron. In vitro studies in mammals demonstrated that linuron competitively inhibits the binding of androgens to the ARs [94] and acts as a weak AR antagonist in transcriptional activation assays [95]. Additionally, prenatal in vivo exposure to high doses of linuron caused reduced testosterone production, altered expression patterns in gene involved in tissue morphogenesis, and morphological disruptions to androgen-organized tissues [96–98]. It is currently hypothesized that antiandrogenic pesticides such as vinclozolin or linuron act through a mixed mode of action including both AR antagonism and reduced testosterone production.

The European Community banned the use of anabolics in Europe by means of laws 96/22/EC and 96/23/EC. Despite these regulations, in many countries, exogenous sex hormones are widely and illegally used in livestock for anabolic purposes during the last 2 months of the fattening period. Such deliberate action raised ovarian cancer incidence in both adult and young animals [99]. Literature search reveals a positive correlation between steroid hormone abuse and cancer incidence [100]. Sex hormones and gonadotropins are responsible for the regulation of granulosa cell proliferation and their physiological changes with maturation [101]. They stimulate cell growth, even in mutated cells, and this is why they are considered cocarcinogens. Thanks to their ability to stimulate mitosis, thus increasing the number of cell divisions, steroids also increase the risk of mutations [102]. Generally, some mutations can be corrected by cellular DNA repair mechanisms, but since these processes require prolonged times, it is believed that faster cell division increases the risk of mutations that can be transferred to daughter cells. Consequently, these hormones may act not only as cocarcinogens but also as true carcinogens, being able to provoke an increased risk for mutation in their target cells. They also stimulate the divisions of the mutated cells [103]. An increased proliferation rate observed in many cell lines indicates that sex steroid hormones act as growth factors and activate respective signaling pathways [104]. Although this is not a uniform view, it seems that sex steroids interfere with mechanisms controlling apoptotic cell death. Regarding androgens, in some experiments, they have been shown to promote granulosa cell apoptosis [105], while other authors have affirmed that they preserved granulosa cells and follicles from undergoing programmed cell death [106]. Today, there is more than 100 varieties of AAS that have been developed, with only a few approved for human or veterinary use. They are used not only by athletic competitors and sportsmen but also by people wanting to alter their physical appearance usually based on the widespread belief that strong, muscled body is the model for the ideal. Some anabolic substances, i.e., testosterone propionate, boldione, or nandrolone, are openly available on the Internet for use by body builders. The International Agency for Research on Cancer classifies them as probable human carcinogens, with a carcinogenicity index higher than that of other androgens such as stanozolol, clostebol, and testosterone [107]. Recently, several models of primary granulosa cell cultures, originating from different animal species, have been devised and are being used to test the effects of EDCs (including anabolic steroids) on cell proliferation, steroidogenesis, and neoplastic transformation [108]. Moreover, after in vivo exposure of an animal to testosterone propionate, an increase in primary follicle number

together with a decrease in those with antrum was observed, leading to the higher proportion of atretic follicles and the lack of corpora lutea within the ovaries [109]. Following these considerations, it should be useful to evaluate the possible involvement of anabolics in the follicular cell transformation being this the first step of carcinogenesis. It might be also possible, in view of the way in which steroids and their derivate act in the mammalian ovary, to check if anabolics trigger follicular cell apoptosis, thereby causing PCOS.

5. Conclusions

In the last decades, it was proven that environmental chemical compounds exert toxic and genotoxic effects and thus form a serious threat to mammalian reproduction. However, the impact of anabolics on ovarian function has been less realized and studied. Recognition and evaluation of risk associated with the AAS use are of the utmost importance for human health. Harmful effects of compounds with antiandrogenic activities acting during folliculogenesis have been shown to affect oocyte survival and follicle growth, as well as steroidogenesis. Better understanding of the mechanisms underlying the consequences of the EDC exposure is required to implement a risk reduction measures to the health of living organisms and, more generally, for a more effective environmental protection activities from chemical pollutants.

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Conflict of interest

Authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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